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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Anmelder:
Applicant(s):
Demandeur(s):

Academisch Ziekenhuis bij de Universiteit van Amsterdam
1105 AZ Amsterdam
NETHERLANDS
UNIVERSITEIT TWENTE
NL-7522 NB Enschede
NETHERLANDS

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Purification, characterization and antibacterial activity of thrombocidin-1 and thrombocidin-2

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Further applicants:
Nederlandse Organisatie voor Wetenschappelijk Onderzoek
2593 BM Den Haag
NETHERLANDS

A PURIFICATION, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF THROMBOCIDIN-1 AND THROMBOCIDIN-2

A1-Purification of thrombocidin-1 and -2 from human thrombocytes.

A1.1-Isolation of granule proteins from thrombocytes.

Buffy coats of human blood from healthy subjects was obtained from the Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands. Eight buffy coats were pooled in a transfer bag (NPBI, Emmer-Compascuum, The Netherlands) (ca. 550 ml), and 200 ml of PBS + 0.38% tri-sodium citrate (w/v) was added. The bag was blown tight with air and centrifuged for 5 min at 600 g and 20°C. The upper phase, containing mainly platelets, was transferred to a new transfer bag. To this platelet concentrate, 1/9 volume of citrate solution was added [75 mM trisodium citrate; 38 mM citric acid]. The bag was blown tight again, and was centrifuged for 10 min at 1750 g (20°C) to pellet platelets. Platelets were resuspended in the same bag in Tris-citrate [63 mM Tris-HCl; 95 mM NaCl; 5 mM KCl; 5mM EDTA; pH 6.8] by gentle massage, and kept overnight shaking at 22°C.Then, platelets were collected in a siliconized flask and the transfer bag was washed with Tris-citrate. Processing of 48 buffy coats routinely yielded ca. 75 ml of highly concentrated platelet suspension containing <0.05% residual leucocytes.

To isolate platelet granules, the platelet concentrate was cavitated three times under nitrogen at 60 atm in a Parr cavitation chamber, and cavitate was collected in siliconized 50 ml tubes (Falcon). This resulted in ca. 90% homogenization of the platelets as determined by Coulter counting. Intact platelets and platelet ghosts were removed by centrifuging the cavitate at 5000 g for 20 min. The supernatant was collected and centrifuged at 12000 g for 20 min, yielding the granules in the pellet. The pellet was resuspended in 5% acetic acid, and sonicated for 30 seconds (pulsed) on ice to rupture granules. The sonicate was kept at 4°C for 24 hours, and subsequently was centrifuged at 125000 g. The supernatant containing granule protein was dialyzed against 5% acetic acid.

A1.2-Purification of thrombocidin-1 and -2.

A rapidtwo-step purification protocol was used for the purification of TC-1 and TC-2 from platelet granule protein: i) cation exchange chromatography, and ii) preparative acid urea polyacrylamide gel chromatography (AU-PAGE), to yield highly purified protein preparations.

A1.2.1-Cation exchange chromatography.

As an ion exchange matrix, CM-sepharose 25 (Pharmacia) was used; phosphate buffer (50 mM, pH 7.0) was used as the mobile phase. A 25 ml sample, containing 3.5 mg/ml of granule protein from approximately 40 buffy coats, was loaded at 0.8 ml/min. Subsequently, the column was washed with phosphate buffer, and protein was eluted with a salt gradient from 0 to 1 M NaCl. Fractions were collected, dialyzed and assayed for the presence of antibacterial proteins by running two separate acid urea gels in parallel. One gel was silverstained (fig 1A), the other gel was used in an overlay assay to detect antibacterial activity (fig 1B). *E.* coli ML35 was used as the test organism. Selected fractions were analyzed by tricine gel electrophoresis to estimate molecular weights of the (partially) purified protein (fig. 2).

The activity present in the starting material (fig 1, cav) is eluted in fractions 35 through 75. Major antibacterial activity can be assigned to two proteins, the most cationic protein is designated as thrombocidin-1 (TC-1), the slightly less cationic as thrombocidin-2 (TC-2). These proteins migrate in an SDS gel as proteins with an apparent molecular weight of 5.5 and 6.5 kD, respectively (fig 2).

A1.2.2-Continuous AU-PAGE.

Fractions eluted from the CM-sepharose column containing antibacterial protein (30 through 75) were pooled, lyophilized, and subjected to a second purification step utilizing continuous gel electrophoresis. Cylindrical gels (3,7x6 cm, 12.5% acrylamide, 3M urea, 5% acetic acid) were poured in a model 491 Prep Cell (BioRad, Veenendaal, The Netherlands) and polymerized at 37°C. Prerunning was at 200V for 2 h. Sample (max. 450 µl) was electrophorized at 40 mA. Protein was eluted in 5% acetic acid at 1 ml/min and collected in 5 ml fractions.

Again, fractions were analyzed in two urea gels run in parallel, followed by staining or by an assay for antibacterial activity (fig 3). TC-1 and TC-2 could effectively be separated (fig 3A). Both proteins have considerable activity against *E. coli* ML35 (fig 3B). Purified TC-1 and TC-2 were lyophylized and redissolved in 0,01% acetic acid, and stored at -20°C until further analysis.

A2-Structure of thrombocidin-1 and -2.

Purified TC-1 and TC-2 were partially sequenced N-terminally by Edman degradation. For both proteins, this N-terminal part showed 100% homology with internal sequences of platelet basic protein (PBP). This indicated that TC-1 and TC-2 are alternative proteolytic degradation products of PBP in addition to the already known PBP-derived peptides connective tissue activating protein-III (CTAP-III), β-thromboglobulin (β-TG) and neutrophil activating peptide-2 (NAP-2). Mass spectrometric analyses were performed to investigate whether the C-terminal parts of TC-1 and TC-2 are identical to the PBP terminus. Based on the amino acid composition of PBP, the calculated masses of TC-1 and TC-2 are 7423,8 and 9287,8, respectively. For TC-1 this mass was confirmed by electrospray mass spectrometry. Electrospray analysis of TC-2 revealed a molecular weight of 9100,5 which was confirmed by MALDI time of flight spectrometric analysis. This difference between calculated and experimental values indicate that, compared to PBP, TC-2 lacks the two C-terminal residues. The calculated mass (9101,4) would in this case be in good accordance with the experimental values. The total amino acid sequences of TC-1 and TC-2 are shown in fig.4.

A3-Structure-function relation of TC-1 and TC-2.

Analysis of TC-1 and TC-2 in a non-reducing tricine gel revealed that the migration of both peptides was retarded compared to their reduced forms. This indicated that TC-1 and TC-2 contain disulfide bridges. To investigate whether the absence of disulfide bridges influenced antibacterial acivity, reduced and non-reduced TC-2 were run on an acid urea gel. To ensure that after reduction refolding of the protein would occur, a sample was included of TC-2

which was reduced and subsequently carboxymethylated with iodoacetamide. Staining of the gel revealed that also in acid urea gels reduction of TC-2 results in retardation of migration. Overlay analysis f a gel run in parallel showed that reduction and carboxymethylation of TC-2 does not reduce, and even seems to increase, antibacterial activity against *E. coli* (fig 5). This offers the possibility that linear peptides derived from TC-1 and TC-2 contain the antibacterial activity.

Thus, the presence of disulfide bridges in TC-1 or TC-2 is not a prerequisite for antibacterial activity.

A4-Antibacterial spectrum of TC-1 and TC-2

Gel overlay and radial diffusion assays with crude platelet granule protein or purified TC-1 and TC-2 revealed antibacterial activity against various micro-organisms such as strains of *Escherichia coli*, *Bacillus subtilis*, *Streptococcus sanguis*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus* including a methicillin resistant clinical isolate (MRSA).

B RECOMBINANT PRODUCTION AND ANTIBACTERIAL ACTIVITY OF PLATELET BASIC PROTEIN (PBP), CONNECTIVE TISSUE ACTIVATING PROTEIN-III (CTAP-III), NEUTROPHIL ACTIVATING PEPTIDE-2 (NAP-2) AND THROMBOCIDIN-1 (TC-1)

B1-Production of recombinant (r)PBP, rCTAP-III, rNAP-2 and rTC-1.

B1.1-Cloning of PBP, CTAP-III, NAP-2 and TC-1 coding DNA and production of the recombinant proteins.

From a human bone marrow cDNA library (Clontech, Palo Alto, USA) DNA coding for PBP was amplified in a PCR.

5'TATAGGATCCATGAGCCTCAGACTTGATACCACC-3' and 5'TATAGGATCCTCAATCAGCAGATTCATCACCTGCCAAT-3' were used as forward and reverse primers, respectively. BamHI restriction sites (underlined) were added to allow cloning in a suitable vector. A stop sequence (boldface) was included to allow proper transcription termination at the stage of protein expression. This PCR was performed using 2 ng of template DNA and Pfu DNA polymerase, which has proofreading capacity. The resulting product was of the expected size (400 bp). This product served as a template in a second PCR to produce the actual TC-1, CTAP-III, NAP-2 and PBP coding DNA to be cloned in expression vectors. For all products, the reverse primer was the same as the reverse primer described above. The forward primers were as follows: for PBP 5'GTGTAACATATGTCCTCCACCAAAGGACAAAC-3'; for CTAP-3 5'GTGTAACATATGAACTTGGCGAAAGGCAAAGAG-3'; for NAP-2 5'GTGTAACATATGTATGCTGAACTCCGCTGCATG-3'; and for TC-1 5'GTGTAACATATGTATCTCCGCTGCATGTGTATAAAG-3'.

NdeI restriction sites (underlined) were included to allow cloning into the vectors, The PCR products were digested with NdeI/BamHI and ligated into a pET 9a (PBP and CTAP-III) or a pET16b vector (NAP-2 and TC-1), linearized with NdeI and BamHI. Constructs were transformed to *E. coli* DH5α, and plated on LB/kanamycin (50 μg/ml) (pET9a) or LB/ampicillin (50 μg ml) (pET16b) plates. Sequencing of cloned DNA confirmed the correct sequence in the constructs. Plasmids containing the correct insert were isolated and

transformed to BL21(DE3)lysE cells, and plated on LB plates supplemented with the proper antibiotics. Of each, single colonies were picked, grown and stored in glycerol broth at -70°C until further use.

Cultures of BL21(lysE) cells transformed with the PBP, CTAP-III, NAP-2 or TC-1 containing pET expression vector were grown in LB medium supplemented with the proper antibiotics. Growing cultures with OD_{660} of 0.3 were induced with IPTG (1mM final concentration). After 3 hours of induction, cells were harvested by centrifugation (5 min, 5000g) and lysed in 20mM Tris HCl, pH 8,2 containing 6M guanidine HCl. Cell debris were removed by centrifugation. Supernatants of rCTAP-III and rPBP producing cells were dialyzed against 50mM phosphate buffer, pH 7,0.

B1.2-Purification of rPBP, rCTAP, rNAP-2 and rTC-1.

rPBP and rCTAP were purified by CM-sepharose cationexchange chromatography and continuous acid urea gel electrophoresis, as described for TC- and TC-2 in sections A1.2.1 and A1.2.2. The N-terminal His-tag in rTC-1 and rNAP-2 allowed purification of these proteins using a His binding resin (Novagen).

B2-Structure of rCTAP-III.

Purified rCTAP-III was partially sequenced N-terminally. The sequence obtained was in accordance with the expected sequence.

B3-Antibacterial activity of rPBP, rCTAP and rTC-1.

Antibacterial activity of purified rPBP and rCTAP and of partially purified rTC-1, still containing the His-tag, was assayed in a radial diffusion assay.All three proteins appeared to be active against *B. subtilis* (fig 6) and *E. coli*.

Fig. 1. Analysis of CM-sepharose purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining (1A) or an overlay using *E. coli* as a test organism (1B). cav: crude granule extract (cavitate), starting material for the purification

Fig 2. Analysis of CM-sepharose purified platelet granular protein by tricine SDS-electrophoresis. Silverstained gel. Selected fractions (compare fig. 1) were analyzed.

Fig. 3. Analysis of AU-PAGE purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining (3A) or an overlay using *E. coli* as a test organism (3B). cav: crude granule extract (cavitate). CM: pooled active fractions (30-75) eluted from CM-sepharose column

Fig 4. Total sequences of TC-1 and TC-2. Residues that were sequenced are indicated in boldface. The remaining part of the sequence is based on 100% homology with PBP. C-termini are deduced from mass-spectrometrical data (see text).

Fig 5. Antibacterial activity of TC-2 and reduced TC-2. Panel A: silverstained acid urea gel. Panel B: Overlay of acid urea gel (test organism: *E. coli*). Lanes 1: TC-2. Lanes 2: reduced and carboxymethylated TC-2. Lanes 3: TC-2 reduced by β-mercaptoethanol treatment. Each lane contains approximately 3μg of protein.

Fig 6. Radial diffusion of rCTAP, rPBP and rTC-1 in *E. coli* containing agarose. Clear zones indicate antibacterial activity. The white ring in the rPBP and rTC-1 containing wells is protein precipitate. Each well contains approximately 3μg of protein.

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CLAIM

1. Purified thrombocidin comprising thrombocidin-1 and/or thrombocidin-2 comprising the following amino-acid sequences:

	1 10 20 30 40
TC-1	LRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQ
TC-2	NLAKGKEESLDSDLYAELRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQ

	50 60 70 80
TC-1	VEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
TC-2	VEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDES

said purified thrombocidins exhibiting anti-bacterial activity.

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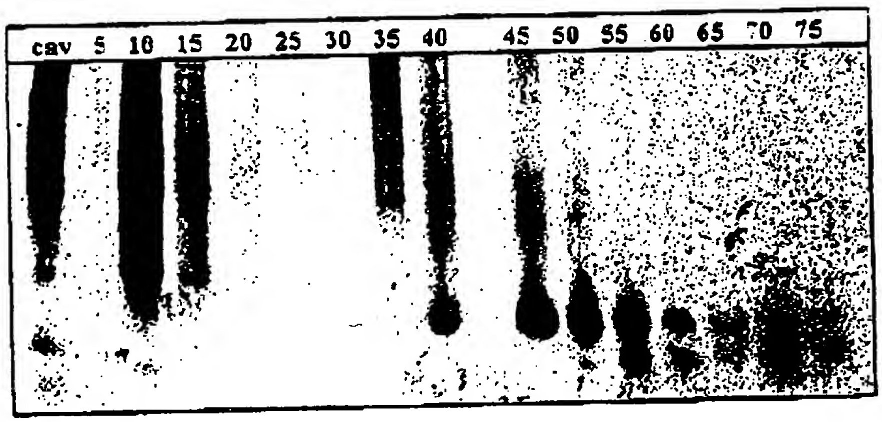

FIG. 1A

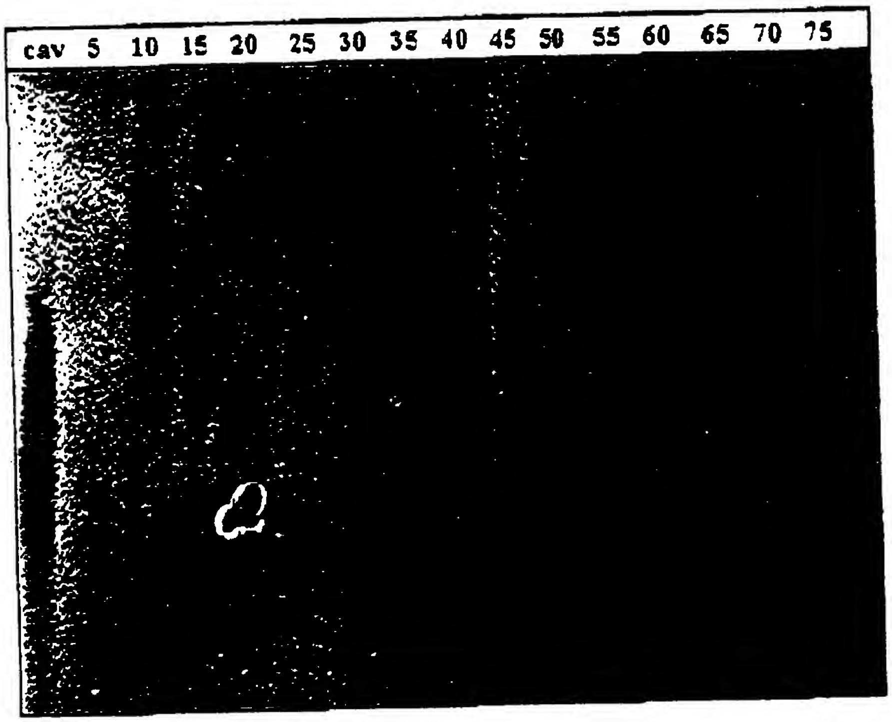

FIG. 1B

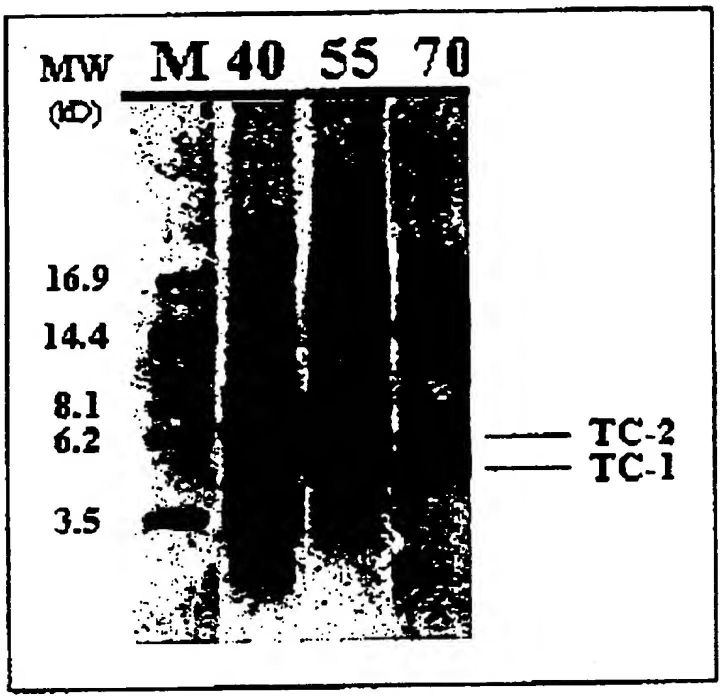

FIG. 2

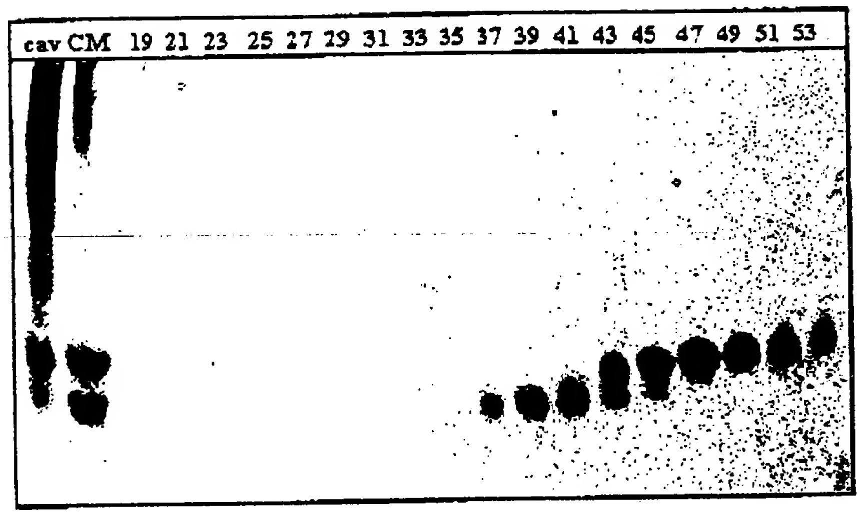

FIG. 3A

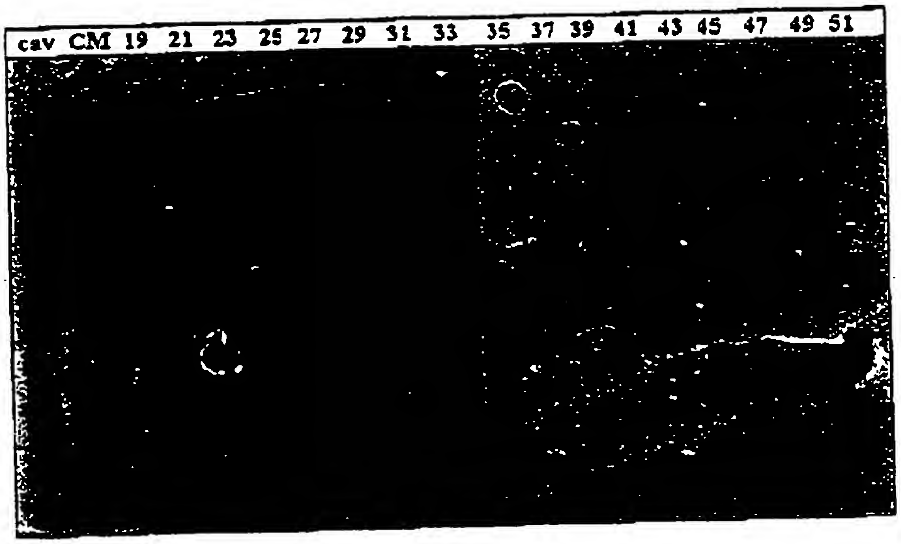

FIG. 3B

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             1         10        20        30        40
TC-1                                LRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQ
TC-2         NLAKGKEESLDSDLYAELRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQ

              50        60        70        80
TC-1         VEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
TC-2         VEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDES
```

FIG. 4

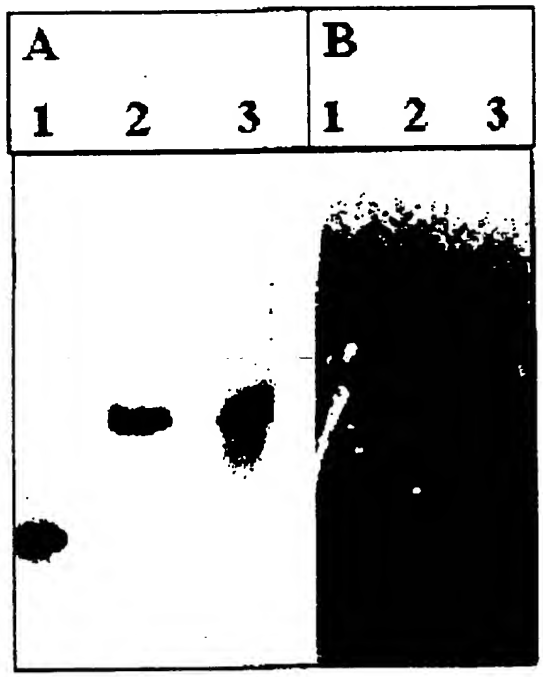

FIG. 5

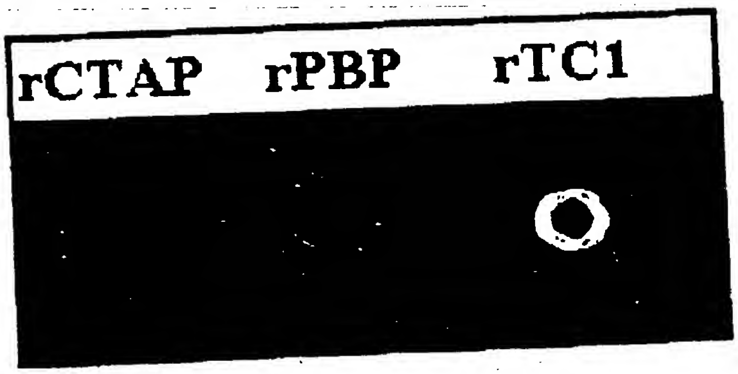

FIG. 6